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☐ Additional inventors are being named on the separately numbered sheets attached hereto.

TITLE OF THE INVENTION (280 characters max)

ASSAY DEVICE AND METHOD

CORRESPONDENCE ADDRESS

CUSTOMER NUMBER:
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ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification - Number of Pages = 31☒ Drawing(s) - Number of Sheets = 5☐ Application Data Sheet, See 37 CFR 1.76☒ Return receipt postcard

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No☐ Yes, the name of the U.S., Government Agency and the Government Contract Number are:☐ Other:

METHOD OF PAYMENT (check all that apply)

☐ A check is enclosed to cover the Provisional Filing Fees.☐ The Commissioner is hereby authorized to charge any additional fees or credit overpayment to Deposit Account 23/2825. A duplicate of this sheet is enclosed.☐ Small Entity Status is claimed.

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Assay Device and Method

BACKGROUND OF INVENTION

5 1. **Field of Invention**

 This invention relates to a method and apparatus for determining an analyte and, in particular, to the determination of a disease condition.

2. **Background of the Invention**

10 An accurate early and ongoing determination of a disease condition is important for the prevention and treatment of human and animal diseases. One class of diagnostic techniques uses immunoassay reactions to detect the presence of either an antigen or an antibody in a sample taken from a subject. These immunoassay methods include, for example, ELISA, immunochromatographic assays (strip tests,
15 dipstick assays and lateral flow assays), and sandwich assays. Accuracy, reliability, and ease of use of these types of assays has improved, but often testing requires laboratory conditions, power supplies, and training that may not be available in some areas where testing is desired.

 One type of sandwich assay uses gold conjugated antibodies to enhance
20 detection. For example, see PCT publication WO/91/01003. Enhancement of a gold colloid signal can be achieved by staining the gold colloids with silver. First, an antigen is immobilized onto a solid polystyrene substrate. A human anti-HIV antibody is then captured by the antigen and is therefore itself immobilized on the substrate. The antibody is then exposed to anti-human IgG labelled with a colloidal gold particle
25 and thus labeled IgG becomes bonded to the antibody. The antigen -antibody-IgG complex is then exposed to a solution containing silver ions and these become nucleated around the gold particles as solid silver particles having a dark color to the eye.

 The development of microfluidics and microfluidic techniques has provided
30 improved chemical and biological research tools, including platforms for performing chemical reactions, combining and separating fluids, diluting samples, and generating

gradients. For example, see US Patent No. 6,645,432, hereby incorporated by reference herein.

SUMMARY OF INVENTION

5 The invention is summarized in the appended claims.

BRIEF DESCRIPTION OF DRAWINGS

 The accompanying drawings are not intended to be drawn to scale. In the drawings, each identical or nearly identical component that is illustrated in various
10 figures is represented by a like numeral. For purposes of clarity, not every component is labeled in every drawing. In the drawings:

DETAILED DESCRIPTION

 This invention is not limited in its application to the details of construction and
15 the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including," "comprising," or "having," "containing", "involving", and
20 variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

 The invention provides a method and apparatus for determining a presence, qualitatively or quantitatively, of a component in a sample. The component may be a binding partner, such as an antibody or antigen, that may be indicative of a disease
25 condition.

 In one aspect, a sample from a subject can be analyzed with little or no sample preparation. The sample may also be obtained non-invasively, thus providing for a safer and more patient-friendly analytical procedure.

 In another aspect, an assay providing high sensitivity and a low limit of
30 detection, comparable to that of the most sensitive ELISA test, is provided. The assay

can be run quickly and results may be permanent, allowing for reading the assay at any time after performing the test.

In another aspect, a sample is flowed over a surface associated with a prospective binding partner of a sample component. The assay can be performed in a channel of a microfluidic device allowing the sample to be flowed over a binding partner, for example, an antigen. Any antigen-antibody complex that forms may be associated with a metal colloid that provides a catalytic surface for the deposition of an opaque material, such as a layer of metal. Therefore, if antibody-antigen binding occurs in the microfluidic channel, the flowing of a metal precursor through the channel can result in the formation of an opaque layer, such as a silver layer, due to the presence of the catalytic metal colloid associated with the antibody-antigen complex. Any opaque layer that is formed in the microfluidic channel can be detected optically, for example, by measuring a reduction in light transmittance through the microfluidic channel compared to a portion of the channel that does not include the antibody or antigen. The opaque layer may provide an increase in assay sensitivity when compared to techniques that do not form an opaque layer.

The term “binding” refers to the interaction between a corresponding pair of molecules that exhibit mutual affinity or binding capacity, typically specific or non-specific binding or interaction, including biochemical, physiological, and/or pharmaceutical interactions. Biological binding defines a type of interaction that occurs between pairs of molecules including proteins, nucleic acids, glycoproteins, carbohydrates, hormones and the like. Specific examples include antibody/antigen, antibody/hapten, enzyme/substrate, enzyme/inhibitor, enzyme/cofactor, binding protein/substrate, carrier protein/substrate, lectin/carbohydrate, receptor/hormone, receptor/effector, complementary strands of nucleic acid, protein/nucleic acid repressor/inducer, ligand/cell surface receptor, virus/ligand, etc.

An “opaque material” is a substance that interferes with the transmittance of light at one or more wavelengths. An opaque material does not merely refract light, but reduces the amount of transmission through the material by, for example, absorbing or reflecting light. Different opaque materials or different amounts of an opaque material may allow transmittance of less than 90, 80, 70, 60, 50, 40, 30, 20, 10

or 1 percent of the light illuminating the opaque material. Examples of opaque materials include molecular layers of elemental metal and polymeric layers.

The term "binding partner" refers to a molecule that can undergo binding with a particular molecule. Biological binding partners are examples. For instance,
5 Protein A is a binding partner of the biological molecule IgG, and vice versa. Likewise, an antibody is a binding partner to its antigen, and vice versa.

"Colloids", as used herein, means nanoparticles, i.e. very small, self-suspendable or fluid-suspendable particles including those made of material that is, e.g., inorganic or organic, polymeric, ceramic, semiconductor, metallic (e.g. gold),
10 non-metallic, crystalline, amorphous, or a combination. Typically, colloid particles used in accordance with the invention are of less than 250 nm cross section in any dimension, more typically less than 100 nm cross section in any dimension, and in most cases are of about 2-30 nm cross section. One class of colloids suitable for use in the invention is 10-30 nm in cross section, and another about 2-10 nm in cross
15 section. Colloids may be associated with a binding partner, for example, an antibody. As used herein this term includes the definition commonly used in the field of biochemistry.

As used herein, a component that is "immobilized relative to" another component either is fastened to the other component or is indirectly fastened to the
20 other component, e.g., by being fastened to a third component to which the other component also is fastened, or otherwise is transitionally associated with the other component. For example, a signaling entity is immobilized with respect to a binding species if the signaling entity is fastened to the binding species, is fastened to a colloid particle to which the binding species is fastened, is fastened to a dendrimer or polymer
25 to which the binding species is fastened, etc.

"Signaling entity" means an entity that is capable of indicating its existence in a particular sample or at a particular location. Signaling entities of the invention can be those that are identifiable by the unaided human eye, those that may be invisible in isolation but may be detectable by the unaided human eye if in sufficient quantity
30 (e.g., colloid particles), entities that absorb or emit electromagnetic radiation at a level or within a wavelength range such that they can be readily detected visibly (unaided

or with a microscope including an electron microscope or the like), optically, or spectroscopically, entities that can be detected electronically or electrochemically, such as redox-active molecules exhibiting a characteristic oxidation/reduction pattern upon exposure to appropriate activation energy ("electronic signaling entities"), or the like. Examples include dyes, pigments, electroactive molecules such as redox-active molecules, fluorescent moieties (including, by definition, phosphorescent moieties), up-regulating phosphors, chemiluminescent entities, electrochemiluminescent entities, or enzyme-linked signaling moieties including horseradish peroxidase and alkaline phosphatase. "Precursors of signaling entities" are entities that by themselves may not have signaling capability but, upon chemical, electrochemical, electrical, magnetic, or physical interaction with another species, become signaling entities. An example includes a chromophore having the ability to emit radiation within a particular, detectable wavelength only upon chemical interaction with another molecule. Precursors of signaling entities are distinguishable from, but are included within the definition of, "signaling entities" as used herein.

As used herein, "fastened to or adapted to be fastened", in the context of a species relative to another species or to a surface of an article, means that the species is chemically or biochemically linked via covalent attachment, attachment via specific biological binding (e.g., biotin/streptavidin), coordinative bonding such as chelate/metal binding, or the like. For example, "fastened" in this context includes multiple chemical linkages, multiple chemical/biological linkages, etc., including, but not limited to, a binding species such as a peptide synthesized on a polystyrene bead, a binding species specifically biologically coupled to an antibody which is bound to a protein such as protein A, which is attached to a bead, a binding species that forms a part (via genetic engineering) of a molecule such as GST or Phage, which in turn is specifically biologically bound to a binding partner covalently fastened to a surface (e.g., glutathione in the case of GST), etc. As another example, a moiety covalently linked to a thiol is adapted to be fastened to a gold surface since thiols bind gold covalently. Similarly, a species carrying a metal binding tag is adapted to be fastened to a surface that carries a molecule covalently attached to the surface (such as thiol/gold binding) which molecule also presents a chelate coordinating a metal. A

species also is adapted to be fastened to a surface if a surface carries a particular nucleotide sequence, and the species includes a complementary nucleotide sequence.

A microfluidic device of the invention can be fabricated of a polymer, for example an elastomeric material such as poly(dimethylsiloxane) (PDMS) using rapid
5 prototyping and soft lithography. For example, a high resolution laser printer may be used to generate a mask from a CAD file that represents the channels that make up the fluidic network. The mask may be a transparency that may be contacted with a photoresist, for example, SU-8 photoresist (MicroChem), to produce a negative master of the photoresist on a silicon wafer. A positive replica of PDMS may be
10 made by molding the PDMS against the master, a technique known to those skilled in the art. To complete the fluidic network, a flat substrate, for example, a glass slide, silicon wafer, or polystyrene surface may be placed against the PDMS surface and may be held in place by van der Waals forces, or may be fixed to the PDMS using an adhesive. To allow for the introduction and receiving of fluids to and from the
15 network, holes (for example 1 millimeter in diameter) may be formed in the PDMS by using an appropriately sized needle. To allow the fluidic network to communicate with a fluid source, tubing, for example of polyethylene, may be sealed in communication with the holes to form a fluidic connection. To prevent leakage, the connection may be sealed with a sealant or adhesive such as epoxy glue.

20 In one embodiment, as shown in FIG. 1, a microfluidic device 110 can be used to provide a substrate on which to perform the assay. Methods of manufacturing such a microfluidic device are provided in U.S. Patent Application No. 6,645,432, incorporated by reference in its entirety herein.

A series of microfluidic channels, 120, 122, and 124, can be used to flow
25 sample and metal precursor across the surface 130 of the microfluidic device. A binding partner, for example, an antigen or antibody, may be disposed on surface 130 at portion 140. Portion 140 may include a stripe, as shown, transversing two or more channels. Alternatively, a binding partner may be disposed on a portion limited to a single channel. Multiple binding partners may be disposed in a single channel and
30 may overlap or be segregated from each other. Methods of applying binding partners to the portion of the surface are known.

To run the assay, a sample, such as a biological sample taken from a subject, is flowed through one or more microchannels 120, 122, or 124, in the direction shown by the arrows. The sample may be a liquid sample, but in some embodiments need not be diluted, purified or treated prior to analysis. The sample may be flowed
5 through the microchannel at a rate sufficient to allow a component of the sample to bind with a binding partner immobilized at portion 140. For example, the sample may be flowed at x, y, or z cm/sec. through the channel. By actively flowing the sample through the channel, the reactive portion 140 is repeatedly exposed to components of the sample, improving reaction kinetics and resulting in an increased formation of any
10 binding pairs. After an adequate amount of flow of sample through microchannel 120, e.g., when detectable binding pairs have formed, a fluid containing a metal colloid associated with a second binding partner of the sample component is flowed to the microchannel, allowing the metal colloid to bind with any sample component that has been associated with portion 140 of the microchannel.

15 After the metal colloid has been given the opportunity to bind with any binding partner at portion 140, a metal precursor can be flowed through channel 120 in a similar manner as was the metal colloid. The metal precursor is flowed through the microchannel at a concentration and a rate that allows an opaque layer to be formed wherever a threshold number of metal colloids have been associated with the
20 surface. Thus, if a gold conjugated antibody is used as a metal colloid, a silver nitrate solution may be used to electrolessly deposit a silver layer on the portion of the channel associated with the gold conjugated antibody. At the completion of this portion of the assay, surface 130 of the microfluidic network may include, in successive layers, an antigen such as HIV antigen, a sample component of an HIV
25 antibody obtained from a subject, a metal colloid such as gold-labeled anti-human IgG, and an opaque layer of metal, such as silver, that has been electrolessly deposited on the metal colloid. Rinsing solutions may be flowed through the channel before or after each of the steps.

30 In addition to depositing metal on any metal colloids that may be associated with portion 140 of microchannel 120, the metal precursor may also be deposited on metal that has previously been electrolessly deposited on the gold-conjugated

antibody. In this manner, an opaque material may be formed over some or all of portion 140 allowing for detection by, for example, the unaided eye or an optical detection device. The opaque material may be a continuous material rather than, for example, independent particles, and may include a horizontal dimension that, in a dimension measured in substantially the same plane as surface 130, measures greater than 1 micron, greater than 10 microns, or greater than 100 microns.

After an opaque layer has been formed, detection of the opaque layer, and therefore determination of the presence of a binding partner, may be determined by visually examining the microfluidic device or by using a detector such as an optical detector. One embodiment of an optical detector is depicted in FIGS. 2, 3 and 9. FIG. 2 illustrates microfluidic device 110, as shown in FIG. 1. Also included is light source 210, here an oscillator-modulated laser diode, and a detector 220, such as an optical IC. As illustrated in the schematic diagram of FIG. 3, the detector signal may be amplified and passed through a bandpass filter centered at the same frequency as the oscillator controlling the light source. The output may then be passed to an A/D converter which can then provide an output on a readout, such as an LCD display. Both the light source and the detector may be powered by a 9 volt battery.

In one aspect, the invention provides an apparatus and method for analyzing a sample using continuous flow. Typically, existing methods such as ELISA and other sandwich assays use a 96 well plate, or similar, for containing a sample for the immunoassay. These methods can expose an antibody or an antigen to a sample component in a fluid, but the fluid is not flowed past the antibody or antigen and diffusion is relied on for bringing binding partners into proximity with each other. The present invention may allow for increased opportunities for binding of a sample component to a potential binding partner at similar or lower concentrations of sample component than previous methods. By flowing a sample containing one binding partner past a surface presenting the other binding partner, a greater number of potential binding partners are placed in proximity to each other than would occur via simple diffusion. In one embodiment, the sample is flowed through a micro-channel providing the benefits of flowing one binding partner past a second binding partner while requiring a small sample, for example, less than 10 micro liters, less than 1

micro liter, or less than 100 nanoliters of sample. The microchannel may be of a material transparent to light that is used to detect the formation of an opaque material in the channel so that any absorbance or transmittance of light through a portion of the channel can be attributed to the formation of an opaque layer.

5 Because immunoassays detect signaling entities, such as enzyme-conjugated secondary antibodies that are dissolved or suspended in a fluid, a relatively long path length is required in order to obtain optimal sensitivity. Thus, one reason why immunoassays have not been applied in microfluidics is the short path length typically presented by microfluidic devices. For example, a microfluidic device may have a
10 thickness of less than 250 microns, less than 100 microns, or less than 40 microns. Therefore, any fluid filling a channel in this microfluidic device would present a perpendicular light pathway of less than 250, 100 or 40 microns. The present method may not be subject to these restrictions because it can use an opaque layer in the solid state, rather than a chromophore in a fluid. The opaque layer may have a thickness of
15 less than 1 micron, of less than 100 nanometers or less than 10 nanometers. Even at these small thicknesses, a detectable change in transmittance can be obtained.

 The geometry of the microfluidic channel may provide for the laminar flow of fluids through the channel, even at relatively high flow rates. Alternatively, turbulent flow may be employed by using even faster flow rates or devices such as microfluidic
20 mixers. Such mixing may provide for a greater amount of contact between potential binding partners.

 The presence, absence, or amount of an analyte in a sample may be indicated by the formation of an opaque material. Although the opaque material may be used to refract light or may be excited to emit light at a similar or different wavelength than
25 the light to which the layer is exposed, the measurement of light transmission may be preferred due to, for example, lower equipment and operating costs, and ease of use. In some microchannels, an opaque layer may be visible to the naked eye and, in particular if reflective, may be detected without the use of instrumentation.

 Any opaque material that forms can be a series of discontinuous independent
30 particles, but in one embodiment is a continuous material that takes on a generally planar shape. The opaque material may have a dimension greater than 1 micron or

greater than 10 microns. The opaque material may be a metal and is preferably a metal that can be electrolessly deposited. These metals include, for example, copper, nickel, cobalt, palladium, and platinum. A metal precursor is a material that can provide the source of the elemental metal for depositing on, for example, a metal
5 colloid. For example a metal precursor may be a metallic salt solution such as silver nitrate. In one embodiment, a metal precursor may include 0.1% silver nitrate, 1.7% hydroquinone and 0.1 M citrate buffer at a pH of 3.5. Some other examples of electrolessly deposited materials can be found in Modern Electroplating, 4th Edition, Schlesinger and Paunovic, Wiley, 2000. Metal precursors can be stored for long
10 periods of time and may be stable for several years whereas optically active compounds may have much shorter shelf lives.

Any metal colloid associated with a surface may be widely scattered over a portion of the surface. For example, gold conjugated antibodies may be bound to sample components that are associated with the portion of the surface but spaces may
15 exist between the gold conjugated antibodies, making them discontinuous. When a metal precursor is first exposed to these gold conjugated antibodies, the precursor may form particulates centered around individual metal colloids. As metal, e.g., silver, is deposited on these metal colloids, the particles become larger and soon the metal precursor may deposit metal not only on gold colloids but on metal that has been
20 previously electrolessly deposited. For example, a silver nitrate solution may deposit silver metal on to silver metal particles that have previously been deposited on gold conjugated antibodies. Thus, as the silver layer continues to grow on silver, as well as on gold, areas that previously were independent particles or islands of metal can join to form a larger, continuous opaque material that can be easily detected. It has been
25 found that a microfluidic system can provide for a relatively smooth, continuous layer of metal. The opaque material may have a thickness greater than x, y, or z nanometers.

A wide variety of chemical and biological materials may be analyzed by the methods and apparatuses described herein. Analytes may include chemicals such as
30 organic compounds and biological materials such as proteins, peptides, nucleic acids and antibodies.

Analytes include any analyte for which a binding partner can be found.

Analytes that may be determined include specific proteins, viruses, hormones, drugs, nucleic acids and polysaccharides; specifically antibodies, e.g.: IgD, IgG, IgM or IgA immunoglobulins to HTLV-I, HIV, Hepatitis A, B and non A/non B, Rubella,

5 Measles, Human Parvovirus B19, Mumps, Malaria, Chicken Pox or Leukemia; human and animal hormones, e.g.: human growth hormone (hGM, human chorionic gonadotropin WM; drugs, e.g.: paracetamol or theophylline ; marker nucleic acids, e.g.; as for genetic finger printing analysis markers; polysaccharides such as cell surface antigens for HLA tissue typing and bacterial cell wall material. Chemicals
10 that may be detected include explosives such as TNT, nerve agents, and environmentally hazardous compounds such as polychlorinated biphenyls (PCBs), dioxins, hydrocarbons and MTBE. Typical sample fluids include physiological fluids such as human or animal whole blood, blood serum, blood plasma, semen, tears, urine, sweat, saliva, cerebro-spinal fluid, vaginal secretions; in-vitro fluids used in
15 research or environmental fluids such as aqueous liquids suspected of being contaminated by the analyte.

In cases where an antigen is being analyzed, a corresponding antibody can be associated with a surface of a microfluidic channel. If an antibody is the analyte, then an appropriate antigen may associated with the surface. When a disease condition is
20 being determined, it may be preferred to put the antigen on the surface and to test for an antibody that has been produced in the subject. Such antibodies may include, for example, antibodies to HIV.

A biological sample may be obtained noninvasively. The low level of detection capable with the invention allows for the use of samples that typically
25 contain lower concentrations of antigens or antibodies than does blood. For example, useful samples may be obtained from saliva, urine, sweat, or mucus. By allowing samples to be obtained noninvasively, the methods of the invention can provide for increased throughput, safer sampling, and less subject apprehension.

The methods and apparatus of the present invention may be capable of
30 obtaining limits of detection (LOD) comparable to those achievable by immunochromatographic assays as well as ELISA. For example, concentrations

below 1 nM and even in the 100 pM range can be detected. The assay can be qualitative, quantitative, or both. As illustrated in FIG. 4, as the concentration of analyte increases, the apparent absorbance of the opaque material increases accordingly. In FIG. 4, the sample component (analyte) is HIV antigen and the sample is human serum. Different dilutions of these sera are shown and in FIG. 6 the formation of an opaque layer indicates a positive result when compared to control at dilutions of 1 to 10 and 1 to 100. Therefore, in addition to presence/absence type tests, a quantitative test may be provided. Such a quantitative test may be of interest, for example, to those monitoring antibody levels in a patient during treatment.

Sensitivity and Limits of Detection (LOD) of the method compare favorably to that obtainable with various state-of-art ELISA techniques. When compared to ELISA techniques using chemiluminescence, fluorescence and absorbance in assaying rabbit IgG, an embodiment of the invention using silver deposition provided comparable sensitivity and LOD numbers. Sensitivity and LOD were calculated using IUPAC definitions and are provided in Table 1 below. Higher sensitivity numbers indicate greater sensitivity and lower LOD numbers indicate a lower LOD.

Table 1

Method	Sensitivity (normalized)	LOD (pM)
Silver deposition	.08	89
Chemiluminescence	.19	22
Fluorescence	.12	163
Absorbance	.04	55

In another embodiment, an assay is provided that requires less time to run than typical immuno-based assays such as ELISA. For example, using a microfluidic device of the present invention, incubation times for each reagent can be less than 10 minutes. For ELISA techniques using microwells, 1 to 3 hours incubation time is typically required for each reagent. Thus, the present method can provide a 6 to 18 fold decrease in incubation time. A portion of this time savings can be attributed to analyzing a sample directly without needing to purify, dilute or otherwise prepare a

sample. For example, a saliva sample may be flowed across a channel without having been diluted, filtered, separated, or otherwise prepared. From the time a sample is obtained to when results are realized, a total time of less than one hour, less than 30 minutes, less than 20 minutes or less than 10 minutes may be realized. One reason for this increase in speed is an improved rate of binding between binding partners. This can be attributed, at least in part, to the flow system of the invention. Systems relying on diffusion, or capillary action are limited in the number of binding partners that can be exposed to each other over a given time period. Furthermore, as diffusion may be temperature dependent, the present invention, utilizing sample flow, may be more temperature independent than other methods, providing for a more robust assay in the field where temperatures may vary from above 40° C to below 0° C.

In another embodiment, two or more parallel assays may be run. A single sample may be physically split into two or more samples using a microfluidic device. A microfluidic device may have a single input channel that branches into two, three, or more parallel channels. Parallel analysis may be performed at different threshold levels of a similar or identical analyte, or for different analytes at the same or different thresholds. A control may also be performed in parallel. Thus, with a single sample run, a sample can be analyzed for two or more analytes at any number of threshold concentrations. A control may also be run concurrently and may be useful in calibrating and/or verifying the detection method that is used. Once an opaque layer is formed, the assay may be stable for an extended period of time, for example, greater than one month or one year, so that assays may be collected and analyzed or re-analyzed at a later date.

A variety of determination techniques may be used. Determination techniques may include optically-based techniques such as light transmission, light absorbance, light scattering, light reflection and visual techniques. Determination techniques may also measure conductivity. For example, microelectrodes placed at opposite ends of a portion of a microfluidic channel may be used to measure the deposition of a conductive material, for example an electrolessly deposited metal. As a greater number of individual particles of metal grow and contact each other, conductivity may increase and provide an indication of the amount of conductor material, e.g., metal,

that has been deposited on the portion. Therefore, conductivity or resistance may be used as a quantitative measure of analyte concentration.

Another analytical technique may include measuring a changing concentration of a precursor from the time the precursor enters the microfluidic channel until the time the precursor exits the channel. For example, if a silver nitrate solution is used, a silver sensitive electrode may be capable of measuring a loss in silver concentration due to the deposition of silver in a channel as the precursor passes through the channel.

Different optical detection techniques provide a number of options for determining assay results. In some embodiments, the measurement of transmission or absorbance means that light can be detected at the same wavelength at which it is emitted from a light source. Although the light source can be a narrow band source emitting at a single wavelength it may also may be a broad spectrum source, emitting over a range of wavelengths, as many opaque materials can effectively block a wide range of wavelengths. The system may be operated with a minimum of optical devices. For instance, the determining device may be free of a photo multiplier, may be free of a wavelength selector such as a grating, prism or filter, or may be free of a device to direct or columnate light such as a columnator. Elimination or reduction of these features can result in a less expensive, more robust device.

In one embodiment, the light source can be pulse modulated, for example, at a frequency of 1,000 Hz. To match the pulse modulated light source, a detector may include a filter operating at the same frequency. By using a pulse modulated light source it has been found that the system can be less sensitive to extrinsic sources of light. Therefore, the assay may run under various light conditions, including broad daylight, that might make it impractical to use existing techniques. Experimental results indicate that by using a pulse modulated light source and filter, results are consistent regardless of the light conditions under which the test is run.

The light source may be a laser diode. For example, an InGaAlP red semiconductor laser diode emitting at 654 nm may be used. The photodetector may be any device capable of detecting the transmission of light that is emitted by the light source. One type of photodetector is an optical integrated circuit (IC) including a

photodiode having a peak sensitivity at 700 nm, an amplifier and a voltage regulator. If the light source is pulse modulated, the photodetector may include a filter to remove the effect of light that is not at the selected frequency.

5

Examples

Example 1

To compare a method of the invention with existing methods, an experiment was designed to assay HIV antibodies using the present method as well as ELISA techniques employing chemiluminescence, fluorescence and absorbance. Procedures and results are described below.

Reagents and equipment were obtained as follows. Rabbit IgG, anti-rabbit IgG (horseradish peroxidase conjugated), anti-rabbit IgG (alkaline phosphatase conjugated), anti-rabbit IgG (gold conjugated), p-nitrophenylphosphate (pNPP), and the silver enhancement kit were obtained from Sigma-Aldrich (St. Louis, MO). AttoPhos was purchased from Promega Corp. (Milwaukee WI). SuperSignal ELISA Femto Max was purchased from Pierce (Rockford, IL). BluePhos phosphatase substrate was purchased from KPL (Gaithersburg, MD). HIV Env antigen (gp41) was purchased from Research Diagnostics (Flanders, NJ). HIV positive serum and control serum were purchased from Golden West Biologicals Inc. (Temecula, CA).

Immunoassays in 96-well microtiter plates were performed using a Tecan Genesis liquid handling robot (Center for Genomics Research, Harvard University). The following Nunc MaxiSorp polystyrene plates were used for the silver reduction and ELISA assays: clear plates for silver reduction and absorbance, black plates for fluorescence and white plates for chemiluminescence. Rabbit IgG (70 μ L for each well) in ten-fold dilutions (10 μ g/mL to 100 pg/mL, which corresponded to 67 nM to 670 fM) was added to the microwells, except for one row to which PBS was added as a negative control; incubation time was 2 hours. Blocking buffer (100 μ L of 0.05% Tween-20 and 1% BSA in PBS) was then added, and left to incubate for 30 minutes. For secondary antibodies, dilutions (30 μ L of 0.05% Tween-20 in PBS) of 1:300 anti-

rabbit IgG (gold-conjugated), 1:1000 anti-rabbit IgG (alkaline phosphatase), and 1:1000 anti-rabbit IgG (horseradish peroxidase) were used; incubation time was 1 hour. For ELISA substrates, pNPP (100 uL; 3 minute incubation), AttoPhos (100 uL, used within 1 week of opening; 10 minute incubation), and SuperSignal Femto ELISA (100 uL; after 5 minutes). For silver enhancement, the solutions of silver and initiator (at 4°C) were mixed in a 1:1 ratio immediately before development; it was filtered through a 0.2 µm filter, and 100 uL was added to each well. After a 20 minute incubation, the silver enhancer solution was removed, and each well was washed with water. In general, warming the silver enhancement solution from 4°C to room temperature increased the rate of silver deposition. In between the addition of each new reagent, each well was washed three times with PBS, with the following exception: deionized water was used to wash the wells after incubation with anti-rabbit IgG (gold) and before silver enhancement, in order to avoid precipitation of AgCl. The plate readers used were Spectramax Plus 384 for absorbance measurements, and Spectramax Gemini XS for fluorescence and chemiluminescence measurements.

The output of the optical IC was light transmittance; apparent absorbance values were calculated using the relation $A = -\log(T / T_0)$, where A is the absorbance, and T and T_0 are the transmission of the light through the sample and reference, respectively, to the photodetector. Air was used as the reference in the plate reader, and a blank polystyrene plate was used as the reference for the portable detector.

The absorbance, fluorescence, and chemiluminescence readings (y) were fit to sigmoidal curves using the software Kaleidagraph and the following equation: $y = Ax^n / (B + x^n) + C$, where x is the concentration of the analyte, and A , B , C and n are floating parameters. Results are illustrated in FIG. 7. This equation describes a general sigmoidal curve with the lowest possible number of floating parameters (four). Curve fitting to all four titrations gave correlation coefficients of over 0.99. The readings y for all four titrations were normalized to the same scale (0 to 1) by linearly transforming each data set to achieve the values of $A = 1$ (asymptote as x approaches infinity) and $C = 0$ (y-intercept).

Limits of detection were calculated according to the IUPAC definition: three times the standard deviation of the blank sample (“noise”) divided by the slope (“sensitivity”). In samples with no rabbit IgG (i.e. negative controls), the methods that exhibited the least to most noise were (after normalization of the signal from 0 to 1): 0.006 for absorbance of pNPP, 0.014 for chemluminescence of SuperSignal ELISA Femto Max, 0.023 for silver (using the portable detector), and 0.066 for fluorescence of AttoPhos. The methods that showed the highest to lowest sensitivities, which were measured as slopes of the best-fit curves in the middle of the linear working range of detection (signal of 0.50), were (in normalized units per 100 pM of analyte): 0.193 for chemiluminescence, 0.121 for fluorescence, 0.078 for silver, and 0.035 for absorbance.

To prepare immunoassay samples for analysis by AFM, holes (4 mm in diameter) were punched in a PDMS slab, and the PDMS slab was placed onto a polystyrene surface. Immunoassays were carried out in individual PDMS wells. After silver development, the PDMS slab was removed, and the samples on the flat polystyrene substrate were analyzed by tapping mode AFM. AFM was performed with a Dimension 3100 Scanning Probe Microscope (Digital Instruments, Santa Barbara, CA) in tapping mode, using silicon probes (Si #MPP-111000; NanoDevices, Santa Barbara, CA) at a scan rate of 0.35 Hz. AFM micrographs are provided in FIG. 8. Streaking was observed for samples with the largest silver grains, which suggested that the silver grains were loosely bound to the surface.

The microfluidic device was fabricated in PDMS using published procedures in soft lithography. The dimensions of the microchannels were 2 mm in width and 130 μm in height. The polystyrene surface was initially patterned with a stripe of HIV Env antigen (10 $\mu\text{g/mL}$) by filling a PDMS channel (conformally sealed onto the polystyrene plate) with the antigen solution. After an overnight incubation, the channel was emptied, the PDMS slab removed from the polystyrene surface, and rinsed the surface with deionized water. The stripe of antigen was covered with an unstructured slab of PDMS, and oxidized the remaining surface of polystyrene with oxygen plasma. After removal of the plasma-protective PDMS slab, another microfluidic channel (also freshly plasma-oxidized) was sealed orthogonally to the

antigen stripe. The dimensions of these microchannels were 2 mm in width and 40 μm in height; the width of the channel must be large enough to register a signal with the portable detector. To avoid sagging of the PDMS, pillars (which took up 12% of the surface area) were included in the channel design. The anti-HIV antibody assay was carried out in the microfluidic channels with the following incubation times: 1 to 4 hours for blocking, 10 minutes for samples, 10 minutes for gold-labelled anti-human IgG, and 13.5 minutes for silver enhancement solution. After 6.5 minutes of silver enhancement, the silver solution was exchanged with a freshly prepared one. The PDMS microchannel was removed above the initial stripe of antigen before measuring the optical density of the silver film. The HIV assay in microwells were performed with the following incubation times: overnight for HIV Env antigen, 2 hours for blocking, 3 hours for samples, 1 hour for gold-labelled anti-human IgG, and 10 minutes for silver enhancement solution.

For each concentration of rabbit IgG and each dilution of human serum, triplicates of the immunoassay were performed, and average values and standard deviations were calculated.

The electronic circuit consisted of a transmitter section and a receiver section. In the transmitter section, a 1 kHz oscillator modulated the light output of a laser diode. A red semiconductor laser diode (Sharp GH06510B2A; normally used for optical data storage applications such as DVD) was used; it emitted at a wavelength of 654 nm with a maximum power of 10 mW. The laser output went through the sample to the receiver section. An optical IC (Sharp IS455; normally used in photocopy machines) to detect and amplify the signal. IS455 provided a linear output current with respect to the input illuminance ($1 \mu\text{A}$ per lux). (The dimensions and costs of the red laser diode and the optical IC were 5.6 mm and \$10, and 5.0 mm and \$2, respectively.) The signal was then filtered by a second-order bandpass filter centered at 1 kHz, and its amplitude registered by a peak detector. The output of the peak detector was connected to an Analog/Digital converter that also encoded the output into binary coded decimal (Intersil ICL7106). The signal was displayed by a 3.5 digit liquid crystal display, which provided an output readout range from 0 to 1999. The entire circuitry was operated with either a 9 V battery or a single polarity 5 V source,

which was inverted with a CMOS voltage converter (Intersil ICL7660) to create a ± 5 V supply. To reduce the noise in the system, pulse modulation of the optical signal at 1 kHz was used to filter the noise power in the frequency spectrum; as a result, only the portion of the optical noise that fit in the pass band of the receiver filter
5 contributed to the overall noise detected. The system could also be used without the signal modulation (i.e. at direct current)

The laser diode and optical IC were placed on two separate circuit boards that were held at a fixed orientation to ensure consistent alignment of the light path from the light source to the photodetector. Between the light source and photodetector, a
10 glass plate was placed. A black transparency, with a pinhole aligned with the light path, was placed on the glass plate to block the transmission of stray light that did not enter the sample. To record a measurement, a polystyrene plate (either a 96-well plate or a plate with a microfluidic device) was placed onto the glass plate. The sample was aligned to the light path by roughly placing the sample over the pinhole, and finely
15 adjusting the x and y position of the polystyrene plate until a maximum transmittance was achieved. The reading from the liquid crystal display was recorded.

To compare two detection methods independent of analyte, microwells of a 96 well plate were subjected to readings by an IC and by a commercial plate reader.

Absorbance of microwells containing different concentrations of BluePhos, which absorbs maximally at 600 nm, as measured by a UV-visible absorbance plate
20 reader and the optical IC described in this study. A direct ELISA was performed on 0.67 pM to 0.67 nM of rabbit IgG as the analyte, using an anti-rabbit IgG conjugated to alkaline phosphatase and BluePhos as the phosphatase substrate. Results are provided in FIG. 10 and FIG. 11. Measurements with both devices were made at 654
25 nm. The best fit line by linear regression is shown (correlation coefficient of 0.998, slope of 1.01, y-intercept of 0.08). Error bars are standard deviations of measurements of three different microwells.

In this assay, in which the colorimetric product is a homogeneous solution in the microwell, the two detection methods resulted in almost perfect agreement
30 (correlation coefficient of 0.998). Thus, inhomogeneity of silver deposition on the surface may have contributed to the imperfect agreement between the two

measurement methods, such that different parts of the same well were sampled by the laser diode and by the plate reader (correlation coefficient of 0.996).

Example 2

5 A schematic representation of one embodiment and an optical detection device is provided in FIG. 9. (A) Red light from the laser diode passes through the silver-coated microwell containing the sample to the optical IC. A pinhole was used to block stray light that did not pass through the sample. The laser diode and the optical IC were driven by the same circuit, which also had an integrated liquid crystal display
10 that showed the measured transmittance value.

Example 3

FIG. 10 provides a comparison of readings of an immunoassay using an optical IC and a UV-visible plate reader. An immunoassay using silver reduction was
15 performed on a 96-well plate that detected rabbit IgG. Optical micrographs of the silver films on microwells are shown for each sample. The apparent absorbance of each microwell was measured by an optical IC, and compared to its reading by a UV-visible plate reader; both measurements were made at 654 nm. The best-fit line by linear regression has a correlation coefficient of 0.989, slope of 1.12, and y-intercept
20 of 0.16.

Having thus described several aspects of at least one embodiment of this invention, it is to be appreciated various alterations, modifications, and improvements will readily occur to those skilled in the art. Such alterations, modifications, and
25 improvements are intended to be part of this disclosure, and are intended to be within the spirit and scope of the invention. Accordingly, the foregoing description and drawings are by way of example only.

What is claimed is:

CLAIMS

Draft claims for H0498.70211us00

- C1. A method comprising:
accumulating an opaque material on a region of a microfluidic chamber;
exposing the region to light; and
determining the transmission of light through the opaque material.
- C1.1 The method of claim C1 wherein the opaque material comprises a metal.
- C1.3 The method of claim C1 wherein determining the transmission of light includes observing the opaque material with an unaided eye.
- C1.4
- C2. The method of claim C1 wherein the opaque material has a dimension of at least x microns.
- C3. The method of claim C1 comprising exposing the opaque material to light of a first wavelength and detecting transmission of light of the first wavelength.
- C4. The method of claim C1.1 wherein the metal comprises silver.
- C5. The method of claim C1.1 wherein the opaque material is formed by electroless deposition.
- C6. The method of claim C5 wherein the opaque material is electrolessly deposited on a metal colloid.
- C7. The method of claim C6 wherein the metal colloid comprises a gold conjugated antibody.
- C8. The method of claim C3 wherein the light is pulse modulated.
- C9. The method of claim C1 wherein the opaque material is deposited over a region having a dimension of at least 10 microns.
- C10. The method of claim C1 wherein the transmittance is determined in the absence of a photomultiplier.

C11. The method of claim C1 wherein the transmittance is determined in the absence of a wavelength selector.

C12. The method of claim C1 wherein the transmittance is determined in the absence of a columnator.

C13. The method of claim C1 wherein the determining step is performed in the absence of line voltage.

C14. The method of claim C1 wherein determining the transmission of light comprises passing light through an optical sample path having a length less than 1 mm.

C15. The method of claim C14 wherein the optical sample path length is less than 100 microns.

C16. C15. The method of claim C14 wherein the optical sample path length is less than 50 microns.

E1. An immunoassay comprising:
a microfluidic chamber having a surface;
at least one of an antigen or an antibody disposed on a portion of the chamber surface; and
an opaque layer associated with the portion of the chamber.

E1.1 The immunoassay of claim E1 wherein the layer is opaque at a wavelength for which the microfluidic chamber is transparent.

E2. The immunoassay of claim E1 wherein the opaque layer comprises metal.

E3. The immunoassay of claim E1 further comprising a layer including a metal colloid.

E4. The immunoassay of claim E1 comprising a plurality of microfluidic chambers.

E5. The immunoassay of claim E1 wherein the layer of metal comprises silver.

E6. The immunoassay of claim E3 wherein the metal colloid comprises a gold conjugated antibody.

F1. A method comprising:

passing a fluid sample over a surface;
allowing a sample component to bind with a binding partner disposed on the surface;

allowing a metal colloid to associate with a sample component; and
flowing a metal solution over the surface to form a metallic layer.

F1. A method comprising:
flowing a fluid sample over a surface;
allowing a sample component to bind with a binding partner disposed on the surface; and
accumulating an opaque material on a portion of the surface.

F1.01 The method of claim F1 wherein the metal colloid associates with a bound sample component.

F1.1 The method of claim F1 wherein the metal colloid comprises gold.

F1.2 The method of claim wherein the metal colloid comprises a gold conjugated antibody.

F1.3 The method of claim F1 wherein the metallic layer is silver.

F1.4 The method of claim F1.3 wherein the metal solution is a silver solution.

F2. The method of claim F1 wherein the metal solution is laminarly flowed over the surface.

F3. The method of claim F1 wherein the surface is a portion of a microfluidic channel.

F4. The method of claim F1 wherein the sample component is one of an antigen and an antibody and the binding partner is the other of the antigen and the antibody.

F5. The method of claim F1 further comprising determining the opacity of the metal layer.

F6. The method of claim F5 wherein determining comprises examining the metal layer with an unaided eye.

F7. The method of claim F5 wherein determining comprises irradiating the metal layer with light and measuring light transmittance.

F8. The method of claim F7 wherein the light is measured at the same wavelength at which it is transmitted.

F9. The method of claim F1 further comprising measuring the conductivity of the metal layer.

F10. The method of claim F1 wherein the fluid is passed over a plurality of surfaces.

F11. The method of claim F10 wherein each of the plurality of surfaces is associated with a different binding partner.

F12. The method of claim F1 further comprising detecting the concentration of metal in the metal solution after flowing the metal solution over the surface.

F13. The method of claim F1 wherein the sample has been obtained non-invasively.

F14. The method of claim F13 wherein the sample comprises saliva.

G1. An assay kit comprising:
a surface including a microfluidic channel;
at least one of an antibody or an antigen associated with a portion of the microfluidic channel;
a metal colloid associated with an antibody or an antigen;
a metal precursor; and
instructions for performing the assay.

G2. The kit of claim G1 wherein the metal precursor comprises a silver salt solution.

H1. A method comprising:
contacting a sample with an antibody or an antigen;
allowing a sample component to bind with the antibody or antigen;
illuminating any bound sample component with a pulse modulated light; and
determining binding of a sample component to an antigen or antibody.

H2. The method of claim H1 wherein the bound sample component associates with a light sensitive moiety.

H3. The method of claim H1 wherein the bound sample component associates with a metal colloid.

H4. The method of claim H1 wherein the sample component is an antibody or an antigen.

H5. The method of claim H1 wherein determining comprises detecting transmission of pulse modulated light.

H6. The method of claim H1 wherein the antibody or antigen is bound to a surface.

H7. The method of claim H6 wherein the surface comprises a portion of a microfluidic channel.

H8. The method of claim H6 further comprising forming an opaque layer on the surface.

H9. The method of claim H8 wherein the opaque layer comprises a metal.

H10. The method of claim H9 wherein the opaque layer is electrolessly deposited on the surface.

H11. The method of claim H10 wherein the opaque layer is deposited on a gold conjugated antibody.

H12. The method of claim H1 wherein the pulse modulated light passes through a sample having an optical path length less than 1 mm.

H13. The method of claim H1 wherein the pulse modulated light passes through a sample having an optical path length less than 100 microns.

H14. The method of claim H1 wherein the pulse modulated light passes through a sample having an optical path length less than 50 microns.

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ABSTRACT

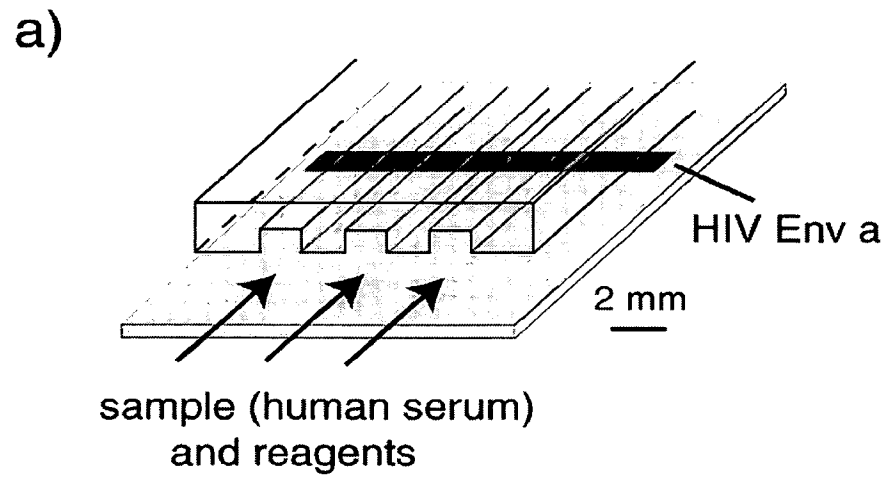


FIGURE 1

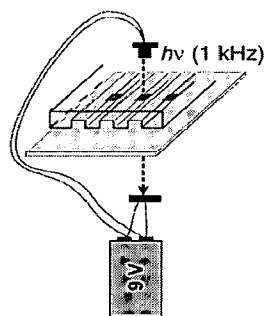


FIGURE 2

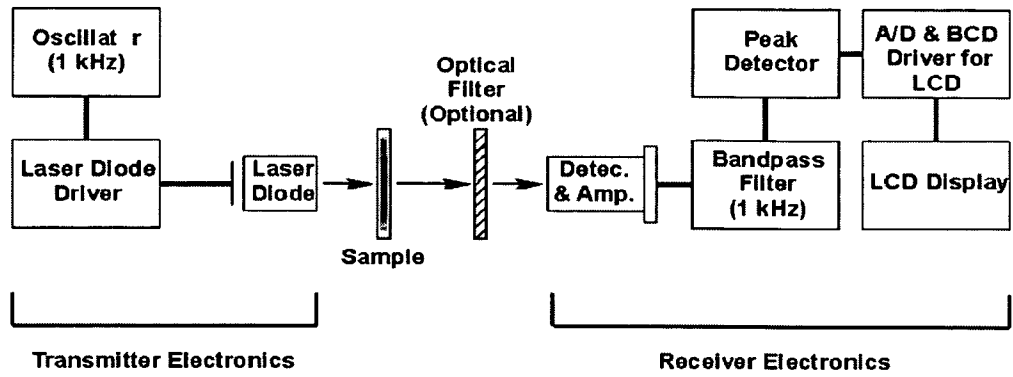


FIGURE 3

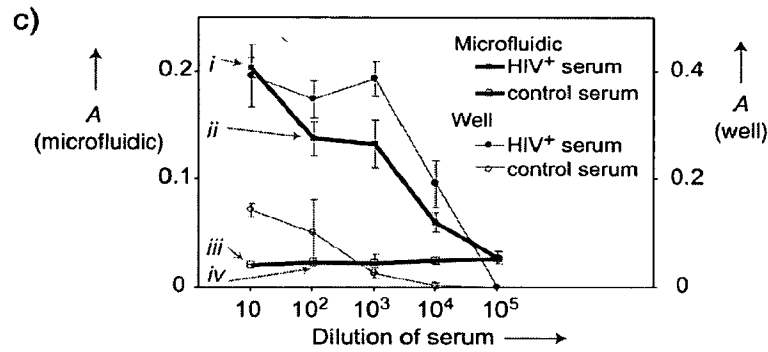


FIGURE 4

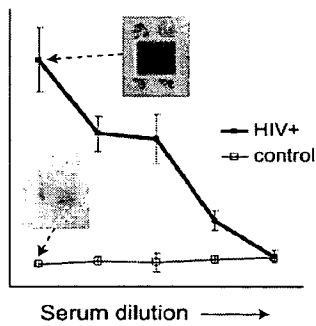


FIGURE 5

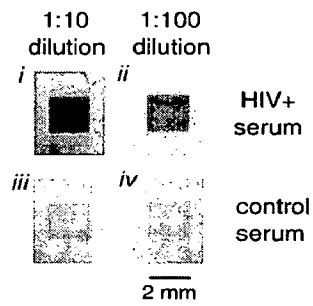


FIGURE 6

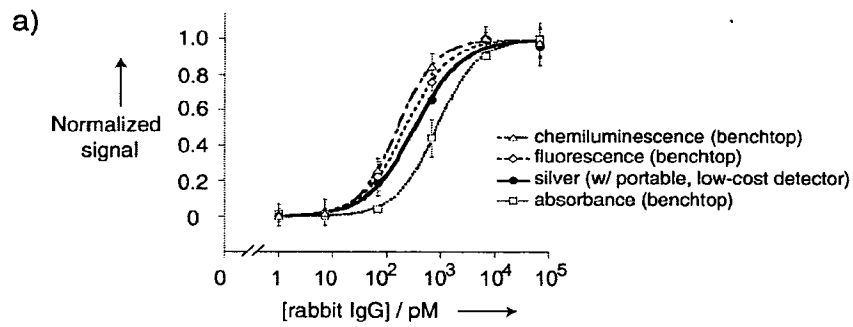


FIGURE 7

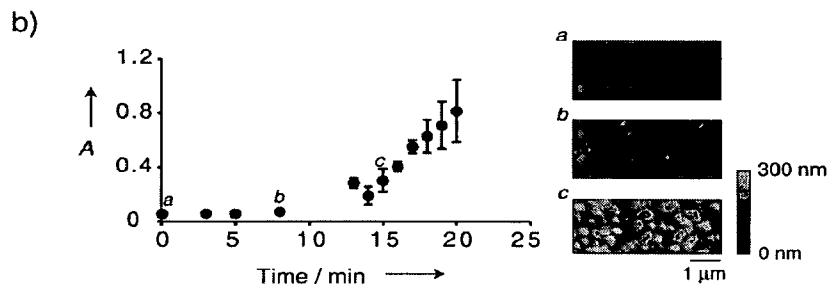
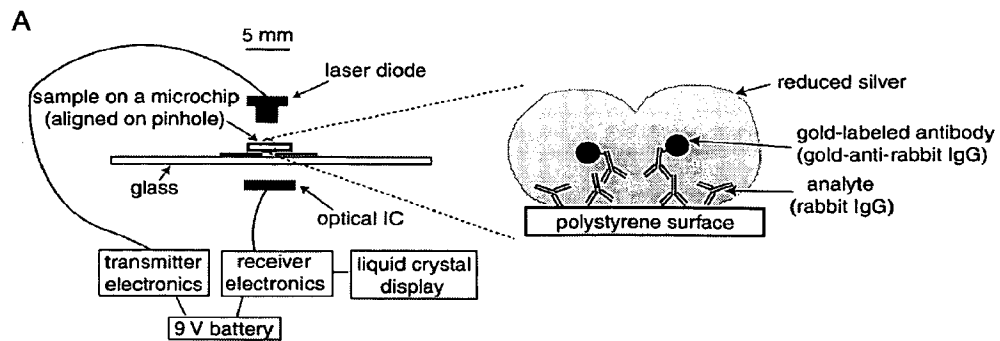
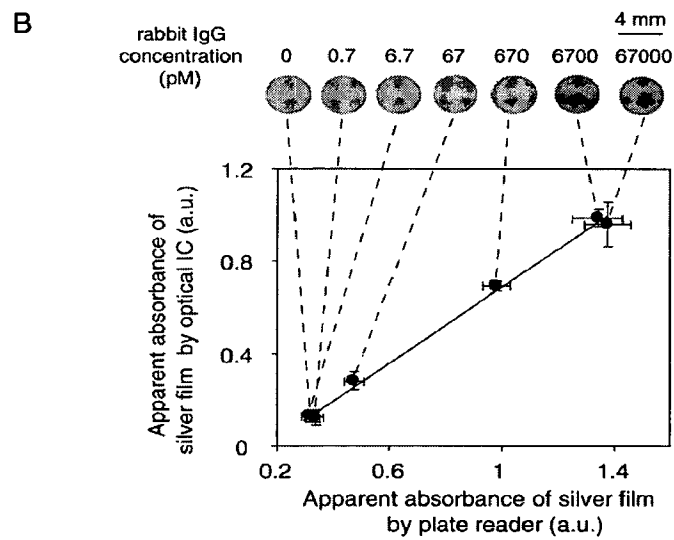


FIGURE 8

**FIGURE 9****FIGURE 10**

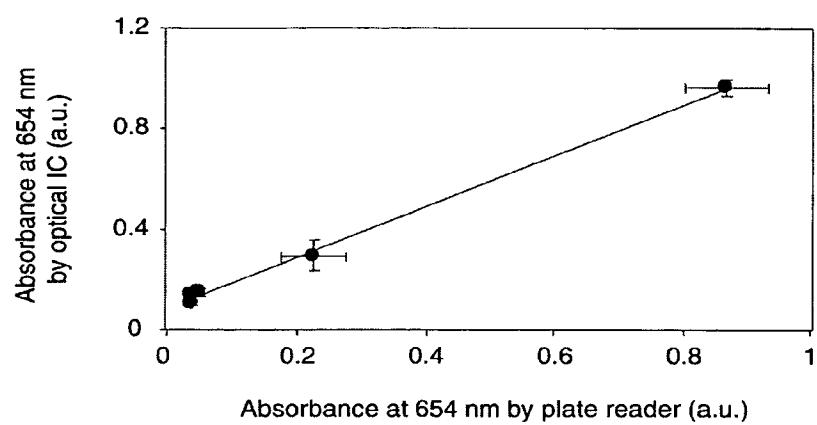


FIGURE 11